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Combination Chemotherapy Using Immune Modulators and Antiviral Drugs Against Togaviruses and Bunyaviruses

Subtitle: Antiviral Therapy Against Banzi Virus, Semliki Forest Virus, and Pichinde' Virus

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We have studied model arboviruses and arenaviruses to develop effective combination therapies using antiviral drugs and immunomodulators. In vivo screening of candidate compounds was undertaken since in vitro screening was not a reliable predictor of in vivo results. The compounds were examined using Pichinde', an arenavirus; Banzi, a flavivirus; and Semliki Forest, an alphavirus. Interferon (IFN), IFN inducers, neutralizing antiserum, ribavirin, interleukin-2 (IL-2), and tumor necrosis factor (TNF) had some prophylactic antiviral action in vivo. Certain combinations of these compounds (e.g., IFN plus TNF, ribavirin or IL-2, and IFN inducers plus neutralizing antiserum) provided greater than additive antiviral effects in vivo. An in vivo model was also developed to study late therapy by starting intervention at the time of viremia when the virus is spreading to target organs. Studies of combination chemotherapy and immunotherapy using the "late therapy" model were undertaken.

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SUMMARY

Many viruses in the families Togaviridae, Flaviviridae, Arenaviridae and Bunyaviridae are important human pathogens. We have studied model arboviruses and arenaviruses to develop effective combination therapies using antiviral drugs and immunomodulators. Since the most powerful combinations will be those that are effective therapeutically, we developed a murine model for testing antiviral regimens after the onset of clinical manifestations of viral disease. We previously reported that *in vitro* screening was not a reliable predictor of *in vivo* results. Thus, *in vivo* screening of candidate compounds was undertaken. Interferon (IFN), IFN inducers, neutralizing antiserum, ribavirin, and tumor necrosis factor (TNF) were all found to have some prophylactic antiviral action *in vivo*. Moreover, certain combinations of these compounds (e.g., IFN plus TNF or ribavirin, interleukin-2 plus IFNα/β, and IFN inducers plus Ig) gave evidence of greater than additive antiviral effects *in vivo*. When the latter combination was used therapeutically, after infected mice had developed measurable fever and/or weight loss induced by Banzi virus, highly significant protection from viral encephalitis was obtained. This model system appears to be a sensitive measure of effective combination therapies.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or service of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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Introduction and Rationale

The research being pursued under this contract has the ultimate goal of designing and identifying effective therapeutic and prophylactic regimes for the treatment of medically and militarily important viral infections. Specifically, we are concerned with Alphaviruses, Flaviviruses, Bunyaviruses, and Arenaviruses. It was the primary goal of the initial contract year to establish test systems in vitro and in vivo to allow us to investigate antiviral agents against representatives of each of these groups. In the second contract year we concentrated our efforts on exploring possible combination antiviral therapies in vivo, using immunomodulators and antiviral compounds which we identified as the most promising candidate antivirals, based on single drug protection experiments. We have focused in vivo work in the second contract year on three virus systems: Pichinde', an arenavirus; Banzi, a flavivirus; and Semliki Forest, an alphavirus. Candidate antiviral compounds which have been the focus of our investigations are interferons, CL246,738, poly I:CLC, ribavirin, immune globulin, tumor necrosis factor, and interleukin 2.

Although prophylaxis of viral infections may be preferable to therapy, prophylaxis is often not feasible when vaccines are not available, when vaccines provide only short duration of immunity, when only a small portion of the population is threatened or when individuals at risk cannot be identified for chemoprophylaxis. Because of these limitations, we have also begun a series of studies designed to establish models of therapeutic intervention in the Banzi and Semliki Forest model systems in mice. In these studies we attempt to achieve late therapy by starting intervention at the time of viremia, when the virus is beginning to spread to target organs. The stage of viremia was selected for the onset on therapy because it causes the first identifiable symptoms of disease that in humans would bring the patient to a physician for treatment. In these experiments, using murine models, the stage of viremia is identified by detection of both virus and accompanying clinical correlates of infection, such as fever and weight loss. We have begun studies of combination chemotherapy and immunotherapy using these model systems.

Experimental Methods

Viruses. Seed Alphaviruses [Semliki Forest (SF), Sindbis (SB)], Flaviviruses [Banzi (BZ), West Nile (WN)] and Bunyaviruses [Bunyamwera (BW), LaCrosse (LAC)] were received from Dr. Robert Shope of the Yale Arbovirus Unit. In vivo experiments were performed using SF (strain original, passage 15) and BZ (strain SA H336, passage 11). Pichinde' virus was received from Dr. David Gangemi, University of South Carolina. The virus (0.1 ml) was inoculated i.p. into 450-500 g Strain 13 guinea pigs. The animals were sacrificed on day 6 post infection, and 10% spleen suspensions prepared. This virus suspension was repassaged in Strain 13 guinea pigs to prepare the final stock virus (passage 14), which was aliquoted and frozen (-70°C).

We noted that after 6-9 months of storage at -70°C, virus stocks begin to lose pathogenicity in vivo. Because of this, BZ and SF viruses are routinely repassaged in newborn mice. Briefly, viruses were inoculated intracranially into suckling mice 1-2 days postpartum. At the first sign of illness, generally 2-4 days after virus challenge, the mice were sacrificed and a 10% brain suspension in Eagles Minimum Essential Media was prepared. The 10% brain suspensions were aliquoted and frozen (-70°C) for future use.

Interferon Assay. All interferon (IFN) preparations were titrated in our laboratory by a plaque-reduction assay in microtiter plates with L-cell and vesicular stomatitis virus as described in our previous report. One laboratory unit/ml of IFN was defined as the concentration of IFN which reduced the number of virus plaques to 50% of the control level. In our system, one laboratory unit corresponds to one international reference unit (IU) (NIH standard G002-904-511).

Antibody Production. Immune globulins were prepared by hyperimmunization of New Zealand white rabbits. Virus stocks (10% brain suspensions) were inactivated by incubation with β-propriolactone (37°, 2 hr), and diluted with Freund's complete adjuvant (1:1). The mixture (1 ml) was injected s.c. into adult rabbits. Antigen boosts were given at days 7, 14, 21 and 28. Serum from immunized rabbits was withdrawn ad libitum, and antigen boosts were given as needed to stimulate production of immune serum.

Animal Models. Protection against arbovirus infections is evaluated in vivo using weanling mouse models. Female outbred mice, strain ICR, at 18-22 g (4-5 weeks) are inoculated with the drug to be evaluated. Route and schedule of drug delivery are dependent on the specific compound being evaluated. Virus challenge (1-3 LD_{75}) is administered intraperitoneally (i.p.). Primary evaluation is by presence of an amelioration of lethal encephalitis. Mice are generally maintained for 14-20 days after virus challenge before an experiment is terminated, but all groups are held for at least 3 days after the last recorded death. Virus control animals die between day 7-9 (BZ) or 5-7 (SF) after virus challenge. Pichinde' virus is administered i.p. to 450-500 g strain 13 guinea pigs. Primary evaluation is by amelioration of lethal hemorrhagic fever. Guinea pigs are maintained for 28-75 days after virus challenge. Virus control animals die between 13-19 days of virus challenge.

Results

Pathogenesis of Pichinde' Virus in Strain 13 Guinea Pigs. We continued our evaluation of the pathogenesis of Pichinde' virus (PIC) infection in strain 13 guinea pigs. Our current focus is on the evaluation of liver function abnormalities associated with PIC infection. Guinea pigs were infected i.p. with 0.1 ml of spleen homogenate, containing 4.5×10^4 PFU PIC; serum was obtained on days 4, 7, 11, and 14 after infection.

We previously reported that prothrombin (PT) and partial thromboplastin (APPT) clotting times were markedly prolonged in infected pigs by day 7. Assays on two clotting factors (Factor VIII and Factor IX) show that levels of these factors decrease significantly within 4 days of virus challenge (10^4 PFU) of strain 13 guinea pigs. Clotting factor levels continue to decrease in concert, reaching 50% of control values by day 11 post infection. All infected animals are dead or moribund by day 14-15 post infection. These results mirror those reported by us for prothrombin and partial thromboplastin clotting times. Results of clotting factor analysis are summarized in Table 1. Preliminary results analysis of factors V, VII, and X are consistent with these findings.

Serum glutamic oxalacetic transferase (SGOT) and serum glutamic pyruvate transferase (SGPT) levels during infection were also determined. Both enzymes show a rise in mean value by day 11 post-infection, and are significantly elevated by day 14. Individual animals show elevated levels as early as day 4,

but on average SGOT and SGPT serum levels rose above normal only during the later part of the infection, which is consistent with the necrosis evident in the liver pathology. Gamma glutamyl transferase (GGT), however, showed an earlier rise (day 4). It subsequently returned to normal or slightly subnormal levels for the course of the infection. The significance of the early rise is unclear, but the normal values later in the infection are consistent with the lack of jaundice in these animals. Alkaline phosphatase levels in uninfected control animals are elevated, which is to be expected in young guinea pigs which are still in the rapid growth phase. By day 4 post-infection alkaline phosphatase levels are markedly decreased, and they remain very low for the remainder of the course of infection. A similar turnoff of alkaline phosphatase has been reported for mice infected with influenza virus and in tissue culture cells challenged with rubella. Liver enzyme studies are summarized in Table 2.

Virus titration studies in tissues of animals infected with 100 PFU of Pichinde' were begun using a plaque formation technique in VERO cells with methyl cellulose, stained with crystal violet after 6 days of cultivation, as we reported previously. Serum viremia levels reached 6.1×10^7 PFU/ml in these studies. Significant virus was also found in liver, spleen, and kidney from infected animals ($0.9\text{-}8.7 \times 10^6$ PFU/0.1g tissue). It is unclear, however, to what extent the virus found in these tissues represent contamination from the blood.

Treatment of Pichinde' Virus Infection with Interferon and Interferon Inducers. Recombinant leukocyte alpha (A) interferon (Hoffmann-LaRoche) is known to be active in guinea pigs, and was used in these experiments. Twenty-nine animals were divided into 4 groups. Drug treated animals received either a low dose (10,000 I.U.) or a high dose (30,000 I.U.) by i.p. injection one hour prior to inoculation of virus. Control groups received virus only, or drug only.

All IFN treated infected animals died between days 13 and 14 after of virus inoculation. The mean day of death for animals treated with 30,000 I.U. IFN was 13.8, for those treated with 10,000 I.U. was 13.8, and for untreated virus infected animals 13.6. The weight curves for treated animals showed the same severe decline as that of untreated infected animals. No difference was apparent between the different IFN dosages. Uninfected drug controls showed no adverse effects, and gained 4% over initial body weight by day 14, as expected in animals of this age.

The IFN inducing agent CL246,738 was tested in two experiments. The combined results were as follows: 30 animals were again divided into 4 groups. Drug treated animals received either low dose (5 mg/kg) or high dose (15 mg/kg) of CL246,738 injected i.p. 24 hours before virus inoculation. Control groups received virus only, or drug only. All infected animals treated with CL246,738 died between days 14 and 16 after infection. Mean day of death for animals receiving 15 mg/kg was 14.4, for those receiving 5 mg/kg 14.6, and for untreated infected animals 15.7. Uninfected animals receiving drug appeared healthy, and gained 1% over initial body weight by day 14.

Treatment of Pichinde' Virus Infection with Ribavirin. Two groups of experiments were performed, using 15 and 12 animals respectively. In the first group of experiments, 8 animals received ribavirin, 25 mg/kg/day i.p. starting on the day of infection, and continuing for 14 days, 4 received virus only, and

3 received drug only. In the second group of experiments, 8 animals received ribavirin treatment which was started on the day of infection and continued for 28 days, and 4 animals received virus only.

When the animals were treated with ribavirin for 14 days, they remained relatively healthy for those 14 days, although a small loss of weight (8% initial weight) can be documented on day 12-14. However, when ribavirin was stopped, the animals became very sick, rapidly lost weight, and all died between days 19 and 28. The mean day of death for treated animals was 22.8.

When animals were treated for 28 days, they appeared entirely well. However, again, some loss of weight (5%) was noted on days 12-14 after infection. Thereafter, they regained the lost weight, and then continued to gain weight. Although they were observed for another 40 days, they showed no illness, and gained weight steadily (Fig. 1). In particular, there were no neurologic symptoms. All uninfected, drug treated controls gained weight as usual, and showed no adverse effects.

Single Drug Titrations In Vivo. Single drug dose response experiments using the Semliki Forest (SF) and Banzi (BZ) model systems were run to confirm previous results and to obtain complete dose response information in our model systems for agents under consideration for use in combination experiments. It is particularly important to obtain information on the end points of single agents which might be used in combination trials so that levels of the antivirals which maximize the likelihood of detecting combinatorial effects are chosen.

CL246,738 was titered to non-protective levels in SF and BZ systems. In both systems oral doses of 0.1 mg/mouse provide 50% protection against a 3 LD_{75} virus challenge. Chloroquine in multiple doses provided only marginal protection against SF infection. This is contrary to our earlier results, where we had found chloroquine to provide significant protection in SF system. Rimantidine did not provide any protection against BZ and SF virus infections.

We noted that BZ was resistant to the protective effect of murine interferon (IFN) α/β when compared to the SF in vivo system. Whereas as little as 300 U of MoIFN-α/β produced significant protection in SF system when administered i.p. 1 h before virus challenge, 10^4 units of the same IFN were unprotective against BZ. By using higher doses, we determined that 3×10^4 - 1×10^5 U of MoIFN-α/β are needed to show consistent protection in the BZ weanling mouse model. Murine IFN-α/β in doses from 30 to 30,000 units per mouse provided significant protection against West Nile virus (Fig. 2). A similar pattern was produced when MoIFN-γ was titrated in the SF and BZ models. Using 3×10^3 units of IFN-γ, 83% protection relative to controls was achieved in the SF system, while no protection was noted at the same levels using BZ.

Dose response data were also extended for the immunomodulator poly I:CLC, and preliminary results with Corynebacterium parvum were obtained. Poly I:CLC consistently gives 50% protection with doses as low as 2×10^{-3} mg/kg total body weight. C. parvum is more effective in the SF than the BZ system if administered either one day prior to challenge (maximizing the IFN response), or 5 days prior to virus challenge (maximizing the natural killer cell response). This differential sensitivity of the SF and BZ model seems to be a consistent feature when IFN and IFN inducers are examined.

Ribavirin was tested in both SF and BZ systems. As expected, no consistent significant protection from lethal encephalitis and no detectable toxicity were demonstrated at drug doses in the 5-40 mg/kg range. When this drug was used in combination therapy, however, we detected a sporadic protection in ribavirin controls in both SF and Banzi (BZ) model systems. Since it has been reported in the literature that ribavirin is not an effective antiviral in a number of arbovirus systems, we extended our initial titration of the drug, using the BZ system. Although there was no significant protection from mortality in these experiments, the results do show that there is a tendency for a greater mean survival time in the treated animals. Interestingly, it is clear that the effect is most marked with a dose of 800 ug/mouse (40 mg/kg) ribavirin (daily, for 5 days), with both lower and higher doses scoring a lower virus rating. This corresponds to the dose included in all combination therapy experiments. The decreasing virus rating which was determined at high ribavirin doses (1200 ug and 1600 ug/mouse daily for 5 days) is probably due to drug related toxicity at high dose levels. Pathologic examination of control mice given the 1600 ug injection course showed definite evidence of pathologic lesions in the liver, although all other organ systems examined seemed normal.

Mechanism of Action of CL246,738. We established in single drug protection experiments that CL246,738 protected mice from lethal challenge with SF or BZ in a dose-dependent fashion, with doses from 15-150 mg/kg being highly effective, and a dose of 5 mg/kg providing partial protection, particularly in the SF system. In order to test whether IFN played a direct role in the protection *in vivo* against SFV and BZV, the same doses of CL246,738 (5-150 mg/kg) used in these *in vivo* experiments were used to induce IFN. Mice were treated orally with the indicated doses of CL246,738. After 24 hours: a) mice were bled from the retro-orbital sinus and serum IFN titers were detected, b) medium (2 ml) was injected into the peritoneal cavity as a lavage and IFN titers in the recovered fluid were determined (titers from the peritoneal lavage represent at least a 40-fold dilution from physiologic levels). CL246,738 induced titers of circulating IFN in a dose-dependent fashion. The same amount of CL246,738 that protected *in vivo* against SFV and BZV also induced significant titers of circulating IFN. A correlation existed between CL246,738-mediated protection of mice from SFV and BZV infection and CL246,738 induction of IFN *in vivo*.

The time course of CL246,738, IFN induction was also studied. CL246,738 was given orally to mice, which were bled from the retro-orbital sinus 6 hour, 24 hour, and 48 hour later. Maximal levels of circulating IFN were achieved 24 hours after administration of C1246,738. Serum IFN levels returned to baseline values by 48 hours after treatment.

We also analyzed the type of IFN produced *in vivo* after CL246,738 treatment. It has been reported that the activation of NK cells by CL246,738 depends upon IFN-α, since antibody to IFN-α/β, but not to IFN-β, completely abolished the effect. In order to test what kind of IFN was induced in the circulation of CL246,738-treated mice, IFN samples of serum and peritoneal lavage were incubated with specific anti-IFN-α, anti-IFN-β, anti-IFN-γ antibodies for 2 hours at 37°C and the remaining IFN activity determined. For both IFN samples, the antiviral activity was totally neutralized by anti-IFN-α antibodies, but not by anti-IFN-β or anti-IFN-γ. Thus, circulating IFN, detected in the serum and peritoneal lavage of CL246,738-treated mice consisted of IFN-α.

We then attempted to find the cell type responsible for the IFN production in vivo after CL246,738 treatment. CL246,738 was given orally to mice and 24 hours later the spleen (SPL), mesenteric lymph nodes (LN), bone marrow (BM), peripheral blood leukocytes (PBL), and peritoneal exudate cells (PEC) were taken from the animals. Cells were prepared as described in Materials and Methods and seeded in culture. Culture supernatants were then tested for IFN activity. The only cell type that produced significant amounts of IFN were the PEC. Interestingly, the titer of IFN produced by PEC inversely correlated with the dose of CL246,738 administered in vivo to the mice. This finding was in contrast to the results obtained with the circulating IFN titers, where a direct dose-response effect was recorded. Moreover, when the IFN produced by PEC was typed, mostly IFN-β was found in the supernatants. These results also contrasted with the results obtained from the typing of circulating IFN.

Thus, IFN was found to be produced by PEC cells in culture after in vivo administration of CL246,738. PEC-produced IFN titers inversely correlated with the dose of CL246,738 given and consisted of IFN-β. None of the cell types isolated produced significant levels of IFN-α, leaving open the question of the origin of the serum IFN in treated mice.

We then attempted to delete the protective effect of CL246,738 by administering antibodies to IFN-α/β or to IFN-β alone in vivo. CL246,738 (15 mg/kg) was given orally to mice 24 hours before challenge with SFV (time 0). Antibodies to IFN were administered intraperitoneally at -24, -1, and +24 hours. Either antibody preparation dramatically reversed the in vivo effectiveness of CL246,738 in protecting mice from SFV infection (Fig. 3). The data indicated, however, that using the same total amount of antibody, the anti-IFN-α/β antibody always neturalized more of the protection effect of CL246,738 than the anti-IFN-β did. This evidence shows that both IFN species play significant roles in the antiviral action of this drug.

Use of Ribavirin in Combination Antiviral Chemotherapy. We have done a series of investigations on the use of ribavirin in combination with IFN or IFN inducers. In all experiments, ribavirin was given as five daily injections, i.p., starting 24 hours before virus challenge. As mentioned earlier, during the course of the experiments we determined that 800 ug/day (40 mg/kg) is the optional ribavirin dose in our murine model systems. In the BZ system, the combination of ribavirin with natural murine IFN α/β consistently produced a greater than additive protection against lethal encephalitis at IFN doses in the range of 10,000-30,000 U/mouse (i.p., 2 hours before virus challenge). This tendency was also seen in the SF system, but did not reach significant levels. No significant enhancement was seen using ribavirin and IFN γ (1,000-3,000 U/mouse) in the BZ system, however. These experiments will need to be repeated using greater amounts of IFN.

Unexpected results were obtained when ribavirin was combined with the IFN α/β inducers. At very low levels of poly I:CLC (0.004- 0.04 ug/mouse, i.p., 6 hours before virus challenge) no consistent interaction of the two antiviral agents was seen in either the BZ of SF systems. At higher doses of poly I:CLC, however, there was clear evidence of antagonism at all dose levels (0.08 - 2.7 ug/mouse) in the BZ system, while the SF results were either neutral or less than additive. As an independent examination of the effect of ribavirin in combination with IFN inducers, this drug was examined in combination with CL246,738 in the BZ system. Slight, but nonsignificant, enhancement of

protection from lethal encephalitis was noted in this experiment at the highest dose of CL246,738 used. We will determine whether the effect is more pronounced at higher doses of CL246,738. We are currently investigating the interaction of IFN inducers with ribavirin in more detail.

Use of Interferon Inducers in Combination Therapy. CL246,738 and poly - I:CLC are both powerful inducers of IFN α/β in the murine system. Both agents also produce more generalized immunomodulation, possibly through their induction of IFN. We investigated these antivirals in combination therapy with each other, antibody, and exogenous IFN. When the two IFN α/β inducers were tested together in the BZ system, the resulting protection from lethal encephalitis was less than the additive effects of the single drug controls. Similarly, poly I:CLC administered in combination with IFN α/β produced less than additive protection in the SF model and slightly negative results in the BZ system. CL246,738 in combination with IFN γ did not depart significantly from additive protection in two experiments utilizing the SF system. Similar results were obtained with IFNα/β in combination with IFN-γ.

Both inducers were also studied in combination with anti-BZ immune globulin. Antibody was produced in mouse ascites fluid as we have detailed previously. In both cases, protection from lethal BZ encephalitis was additive at low inducer doses, and greater then additives at doses of inducer which began to produce significant protection in the single drug controls.

Combination Antiviral Therapy with Lymphokines and Cytokines. We began studies on the use of combinations of lymphokines and cytokines in antiviral therapies, concentrating on combining IFN with tumor necrosis factor (TNF) and interleukin-2 (IL-2). TNF was administered i.p., daily for five days, starting 24 h before virus challenge. TNF alone gave sporadic elevation of the mean day of death (MDD) in both BZ and SF systems, and also produced an inconsistent protection from lethal encephalitis (Table 3). The combination of the Hu rTNFα with IFNα/β consistently increased the MDD, and, at higher doses of TNF (1-3 ug/dose) significantly enhanced protection from BZ lethal encephalitis over the expected additive interaction of single drug controls (Table 4). Interestingly, TNF and IFN γ did not interact positively in the same system.

IFN α/β was also used in combination with either human or murine interleukin 2. In these experiments IL-2 was given i.p. 24 hours before virus challenge, and IFN i.p. at -2 hours. Purified, recombinant human IL-2 (Boerhinger Mannheim) had a slightly protective effect against BZ encephalitis, although protection from mortality with IL-2 alone (1,000 units/mouse) was not seen in every experiment. IL-2 also seemed to enhance the protective effect of IFN α/β, with combined protection from mortality reaching synergistic levels in most experiments (Table 5). This combination effect was not seen when crude human IL-2 was used, possible because of the presence of other, undefined factors in the inoculum. Only purified material will be used in future experiments. When equivalent doses of purified, murine rIL-2 (Genzyme) were used in parallel experiments, erratic results were obtained. Some discoloration of a portion of this material was noted in its preparation, it is possible that the IL-2 was partially inactive. The combination of IL-2 with IFNα/β needs to be explored further, utilizing higher doses and multiple injections of IL-2.

Correlates of Infection in Banzi and Semliki Forest Virus Infected Mice. Experiments were designed to detect easily observable correlates of infection of

arbovirus challenged mice. The rationale was to try to determine whether there were effects of the infectious process which could be used as reliable markers of the course of the infection. Only markers which corresponded to symptoms which might be apparent to an infected individual were considered. Core body temperature, body weight, and motor activity were determined daily for mice challenged with $3LD_{75}$ BZ virus, and for untreated controls. Motor activity was recorded on a seven point scale, with 7 equalling normal behavior and the lowest score (1) representing death. These results show that the earliest detectable change (day 3) in the infected mice is a rise in temperature. A rise in core body temperature to $\geq$ 99.5°F was scored as a significantly elevated temperature. Thus, treatment of BZ infected mice on day 3 (day of first fever) would correspond to the earliest possible therapeutic intervention in a clinical setting when infection times are not known. Parallel experiments with SFV showed that a loss of body weight compared to uninfected animals on day 2 post-infection was the earliest apparent marker. This was followed by the appearance of fever on day 3.

We extended these studies on the disease process in the Banzi and Semliki Forest virus models by determining the levels of virus in the blood and in the target organ (brain) of these encephalitic viruses throughout the course of infection. Briefly, 30-35 animals were infected with $3LD_{75}$ of virus (i.p.) on day 0. Blood samples, weights, and temperatures were obtained daily from groups of five mice throughout the course of the infection; the mice were sacrificed and brains collected immediately after bleeding. Viremia was shown to correlate with our previously determined clinical markers of infection fever (BZ) and weight loss (SF). Brain virus titers reached a peak of 1.25×10^9 PFU/g on day 7 p.i. for BZ, and 2.5×10^8 PFU/g on day 5 for SF (Figs. 4,5).

Late Intervention Antiviral Therapy. Using our model of late intervention, after the onset of clinically detectable correlates of viral infection, we began studies on therapeutic antiviral treatments. In experiments preliminary to combination therapy trials, we attempted to evaluate the protection of IFN alone, in single injections given after virus challenge. These experiments showed that multiple dose IFN therapy will be necessary for late intervention, since even at relatively high doses of IFN no protection was scored when treatment was given as early as 24 hours after virus challenge.

The drug CL246,738 was also evaluated therapeutically, using the SF model. The compound was administered 24 hours after virus challenge, in contrast to the normal procedure of giving this drug 24 hours before the virus. Significant protection was recorded at the highest dose of drug used (3 mg/mouse). Thus, C1246,738 can be an effective therapeutic agent, but much higher levels of the drug must be used when it is administered after virus challenge in order to achieve the same level of protection as seen when the drug is administered prophylactically.

Ribavirin was also evaluated in the BZ late intervention model, alone and in combination with either IFNα/β (10,000 U/mouse) or poly I:CLC (80 ug/mouse). Ribavirin (800 ug/mouse at +72, +96, and +120 hour) had a minimally protective effect when given to BZ-infected mice which had developed fever. Neither combination treatment enhanced this effect. It remains to be seen if continuing the ribavirin therapy past 120 hour will enhance the protection seen.

We have concentrated most of our efforts with the BZ late intervention model on therapy using immune globulin and the interferon-inducing immunomodulator poly I:CLC. When 1-5 daily doses of poly I:CLC (80 ug/mouse) is given to fevered mice starting on day 3 post-infection, no significant protection from lethal encephalitis was recorded, although a single prophylactic dose was highly protective and a combination of prophylactic and therapeutic treatments were completely protective in the same series of experiments. Single day 2 interventions in the SF model were similarly unsuccessful.

Immune globulin to BZ was an effective agent in late intervention treatments of mice both when used alone and in combination with poly I:CLC. Massive doses of antibody (3900 U/mouse) were sufficient to totally protect fevered mice on day 3 after infection in most experiments, and gave partial protection on day 4 post-infection. When combined with a single 80 ug/mouse injection of poly I:CLC on day 4 p.i., there was evidence of enhanced protection against lethal BZ encephalitis. Using slightly lower doses of Ab, enhanced survival in combination treated mice was also recorded for fevered mice on day 3 p.i. (Fig. 6). In general, the combination of immune globulin and poly I:CLC appears to have potential in the late intervention model as long as sufficient Ab is administered to provide a partial response by itself.

Discussion and Conclusions

In our present studies, Pichinde' virus infection in the guinea pig did not respond to therapy with interferon, even when given before infection. We will need to confirm these results by extending the range of IFN administered to infected animals. We will also need to show that IFN is induced by CL246,738 in the guinea pig's system. Nevertheless, our results to date are consistent with previous reports of high resistance of arenaviruses to interferon. Thus, the human arenaviruses may not respond to exogenous administration, or endogenous induction of interferon.

Although it has been reported that ribavirin is beneficial in guinea pigs infected with arenaviruses, there is little specific data available on the course of infection in the treated animals. In our study, treatment for only 14 days blocked onset of illness, but did prevent the appearance of lethal disease. In contrast, when the animals were treated for 28 days, all disease was eliminated. This may be an illustration of the virostatic nature of ribavirin and the dependence on host response for the elimination of the virus. Further work is necessary to determine how late in the course of infection with Pichinde' we can begin antiviral therapy, and still prevent death.

We investigated the antiviral effect of CL246,738 against two arboviruses in vivo. The compound proved extremely potent in protecting outbred weanling mice against lethal encephalitis produced by Semliki Forest virus, an alphavirus, and Banzi virus, a flavivirus. We also investigated the mechanism of this antiviral action. The importance of the direct role of interferon in the antiviral state was evaluated. Our studies demonstrated that CL246,738 was protective at doses which did not induce significant natural killer activity, and which induced little or no circulating interferon. These same doses of the drug did induce peritoneal exudate cells (PEC) to produce significant amounts of interferon, however. Higher doses of the compound produced high levels of circulating IFN. We investigated the nature of this PEC-produced interferon and

its role in the protection of mice challenged peritoneally with a model Togavirus.

The PEC-produced IFN was found to be IFNβ. The dose of CL246,738 administered inversely correlates with the titer of PEC-produced IFNβ. However, CL246,738 induces circulating IFNα at levels which directly correlate with the dose of the compound administered. CL246,738-induced IFN (both α and β) is directly involved in the protection of mice from Semliki Forest and Banzi virus infection. Anti-IFNα/β antibody treatment in vivo totally neutralizes the protective effect of CL246,738 against SF virus. Anti-IFNβ alone is sufficient to neutralize the majority of the in vivo protection of CL246,738 against SF virus. At a dose of CL246,738 which induces detectable, but non-protective, levels of circulating IFNα, the additional IFNβ produced by the peritoneal cells is sufficient to protect mice from challenge with SF virus. Hence, both IFNα and β appear to be involved in the antiviral action of this compound, and IFNβ probably plays the major role in protecting infected animals at low dose levels.

Our experiments showed ribavirin to be a useful antiviral drug against togavirus infections when used in conjunction with other antiviral agents. Ribavirin combined with IFNα/β shows particular promise in the BZ model system, with levels of protection exceeding expectations based on an additive interaction of these two agents. The effect of ribavirin alone in this system is minimal, however. Equivalent protection was not seen when ribavirin was combined with IFNγ, possibly because the doses of IFN used were insufficient. Preliminary experiments using ribavirin with inducers of IFNα/β showed either insignificant interactions or evidence of antagonism. These suprising results need to be investigated in more detail.

Combination antiviral therapy with IFN and the cytokines IL-2 and TNF are summarized in Tables 3-5. TNF alone has recently been shown to have an antiviral action in vitro, which is independent of its lytic function. We were able to demonstrate that model flavi-, alpha-, and bunyaviruses are susceptible to the antiviral action of TNF when measured in HEp-2 cells (Table 6). TNF was only weakly antiviral in vivo, however (Table 3). When combined with IFNα/β, a potent synergistic effect was found in the BZ model system (Table 4). The mechanism of this interaction has yet to be determined.

Hu rIL-2 also produced a positive interaction with IFNα/β in the BZ system (Table 5). IL-2 did not produce a marked antiviral effect when administered alone in the same experiments, however. The levels of IL-2 used in these experiments (a single dose of 1,000 units) were minimal. It will be interesting to examine this interaction in more detail using multiple potent doses of IL-2. We can hypothesize that the interaction with IFNα/β seen in our experiments is through the induction of other lymphokines by the IL-2, presumptively IFNγ. The short half-life of IL-2 in the murine system (2-3 minutes) strongly suggests that its antiviral effect is the result of an induction phenomenon.

As more potent antiviral therapy is developed, it becomes possible to begin treatment of viral diseases after the onset of infection. This has been shown for some viral infections of humans including herpesvirus and respiratory syncytial virus infections. In the case of flavivirus infections, the first signs and symptoms that might bring a patient to a physician for treatment include malaise, fever, and loss of appetite, all of which can occur during the viremic phase of the infection as the virus is spreading to the target organs.

We have modeled such an infection with Banzi virus infection of mice. Our results show that, during the viremic phase of infection, there is a definite and uniform febrile response. Application of combined therapy with high-titered antiserum and a potent interferon inducer resulted in strong protection of the febrile mice against mortality from the subsequent viral encephalitis. In one experiment, 73.3% of the febrile mice whose treatment was initiated on day 3, were protected against mortality from viral encephalitis and 21.4% of those with first treatment on day 4 were protected. The significance of this finding is in its implication for the late therapy of similar human infections. Thus, those diseases that present themselves with fever and malaise during viremia (e.g., many of the other flavivirus infections, togavirus infections, arenavirus infections, and varicella virus infection) might be treatable if suitably effective therapy were available.

Table 1. Summary of Clotting Factor Studies in Strain 13 Guinea Pigs Infected with Pichinde' Virus.

Clotting Factor	Day			
	0	4	7	11
Prothrombin[1]	40	35	124	99
Activated Partial Thromboplastin[1]	25	23	35	38
Factor VIII[2]	100	60	50	44
Factor IX[2]	100	60	60	40

[1]Clotting times (seconds)

[2]Percent of control values

Table 2. Summary of Liver Enzyme Functions in Strain 13 Guinea Pigs Infected with Pichinde' Virus

Enzyme	International Units per ml on day				
	0	4	7	11	14
Serum glutamic oxalacetic transferase	120	130	96	283	581
Serum glutamic pyruvate transferase	44	30	27	50	75
Serum gamma glutamyl transferase	8.5	16.2	8.0	5.2	5.5
Serum alkaline phosphatase	625	100	20	10.0	180

Table 3. Antiviral Action of TNF *in vivo*.

Virus	ug TNF	MDD	% MRT	VR
BZV	0	7.3	100	1.00
	0.1	7.6	100	1.04
	1.0	8.0	90	1.37
BZV	0	8.7	91	1.00
	0.1	9.0	91	1.03
	1.0	10.0	100	0.96
	3.0	8.0	55	1.63
BZV	0	8.5	100	1.00
	1.0	8.0	100	0.94
	3.0	10.8	100	1.27
SFV	0	6.3	90	1.00
	0.1	7.2	90	1.09
	1.0	7.3	70	1.59

TNF was administered i.p. at -24, 0, +24, +48, and +72 hours, relative to virus challenge.

Table 4. In Vivo Protection from Banzi Virus Encephalitis with Hu rTNFα and MoIFNα/β

μg TNF	Units IFN	MDD	% MRT	p Value[1]
1.0	0	8.5	97	NA
	10,000	10.9	36	0.004
	30,000	10.1	80	0.22
	100,000	10.4	80	0.10
3.0	0	9.8	76	NA
	10,000	11.3	24	0.0004
	30,000	11.2	70	0.09
	100,000	13.2	50	0.02
0	10,000	9.0	65	NA
	30,000	11.3	90	NA
	100,000	10.6	80	NA
Virus Control		8.5	97	NA

[1]Fishers' Exact Test, observed % mortality. Expected mortality caclulated as: (% MRT Treatment #1) X (% MRT Treatment #2).

Table 5. In vivo protection from Banzi Virus Encephalitis by Hu rIL-2 and Mo IFN-α/β

Experiment	Units IL-2	Units IFN	MDD	% MRT	pValue[1]
1	10^2	10^3	9.4	62.5	0.03
		10^4	11.0	87.5	0.30
	10^3	10^3	9.4	87.5	0.30
		10^4	9.8	50.0	0.007
2	10^3	10^4	8.7	60.0	0.17
		$3X10^4$	10.3	30.0	0.06
3	10^3	10^4	9.7	50.0	0.24
		$3X10^4$	10.0	16.6	0.03
Virus control			8.2	90-100	____

[1]Fisher's Exact Test, observed % mortality. Expected mortality calculated as (% MRT Treatment 1) X (% MRT Treatment 2).

Table 6. In Vitro Antiviral Activity of rTNFα

Virus	MIC_{50}/ml (PFU)	
	Units	μ Grams
Banzi	460	9.2×10^{-3}
Sindbis	2.65	5.3×10^{-5}
Bunyamwera	516	1.0×10^{-2}

All titrations determined on HEp-2 cells.

FIGURE LEGENDS

Figure 1. Extended Ribavirin Treatment of Strain 13 Guinea Pigs Infected with Pichinde' Virus. Strain 13 guinea pigs (approximately 500 g starting weight) were infected with 0.1 ml spleen homogenate representing 100 PFU Pichinde' virus (passage 15). Treatment with ribavirin (25 mg/kg) was begun on day 0 and continued daily for 28 days. A. Weight gain of ribavirin treated and control animals. B. Mortality. No animals died after day 24.

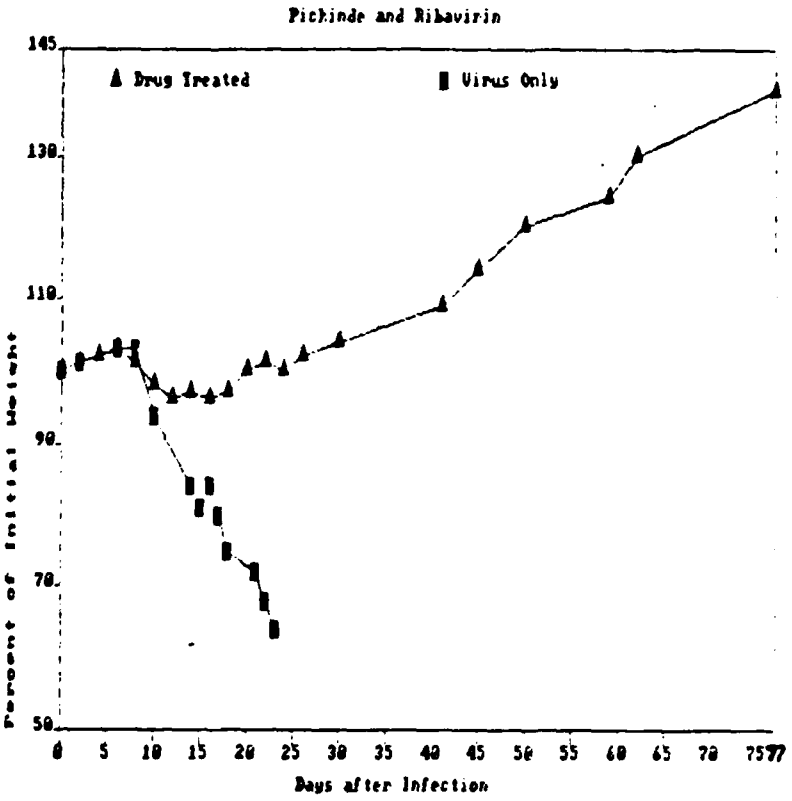
Figure 2. Relative Sensitivity of Model Arboviruses to Interferon In Vivo. Outbred weanling mice (18-20 g) were administered 300 or 10,000 units of natural murine IFNα/β one hour before virus challenge. Mice were given approximately $3LD_{75}$ West Nile, Semliki Forest, or Banzi virus, administered i.p. Mortality of treated and control groups (6-10 mice/group) are shown.

Figure 3. Antibody to Interferon Neutralizes the Antiviral Activity of CL246,738. Outbred weanling mice (7-11/group) were treated with IFNα/β (3,000 units) at -2 h or CL246,738 (0.3 mg) at -24 h, relative to challenge with Semliki Forest virus at time 0. Anti-IFN (1,000 units) was given at -1 and +24 h for the IFN treated mice, and at -24, -1, and +24 h for the CL246,738 treated mice. Anti-a/b = anti-IFNα/β; anti-b = anti-IFN-β.

Figure 4. Viremia in Banzi Virus Infected Outbred Mice. Thirty-five mice were infected with $3LD_{75}$ BZV on day 0. Brains were harvested from mice daily (5/day) to determine virus levels in the target organ. Blood (0.0 ml, retro-orbital sinus) was drawn and temperatures and weights were recorded before the animals were sacrificed. Viremia and virus load in the brain are plotted.

Figure 5. Viremia in Semliki Forest Virus Infected Outbred Mice. Thirty-five mice were infected with $3LD_{75}$ SFV on day 0. Brains were harvested from mice daily (5/day) to determine virus levels in the target organ. Blood (0.1 ml, retro-orbital sinus) was drawn and temperatures and weights were recorded before the animals were sacrificed. Viremia and virus load in the brain are plotted.

Figure 6. Combination Antiviral Therapy With Poly I:CLC and Anti-Banzi Immunoglobulin in a Late Intervention Model. Outbred weanling mice were infected with $3LD_{75}$ Banzi virus. Those mice with elevated temperatures (>95.5°F) 72 h post-infection were divided equally (15 mice/group) and used for therapeutic intervention studies. Poly I:CLC (80 µg/mouse) and anti-Banzi antiserum (3,000 units/mouse) were administered i.p. alone or in combination. All untreated control mice died between days 7-9. Experiment A. Mice were treated at +72 h after virus challenge. Experiment B. Mice were treated +96 h after virus challenge.



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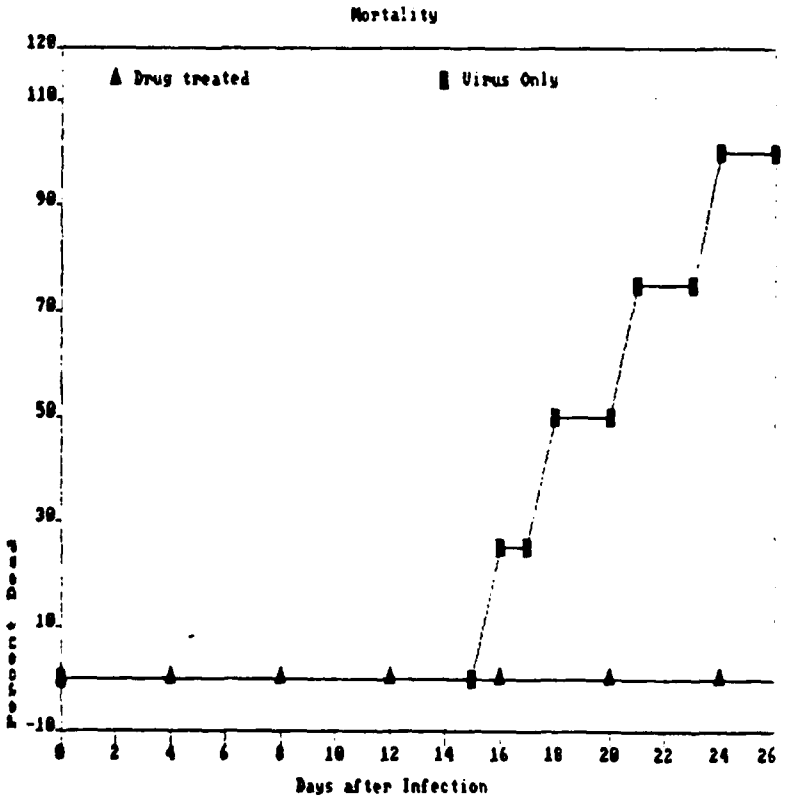

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FIGURE 1. Extended Ribavirin Treatment of Pichinde' Infected Guinea Pigs.

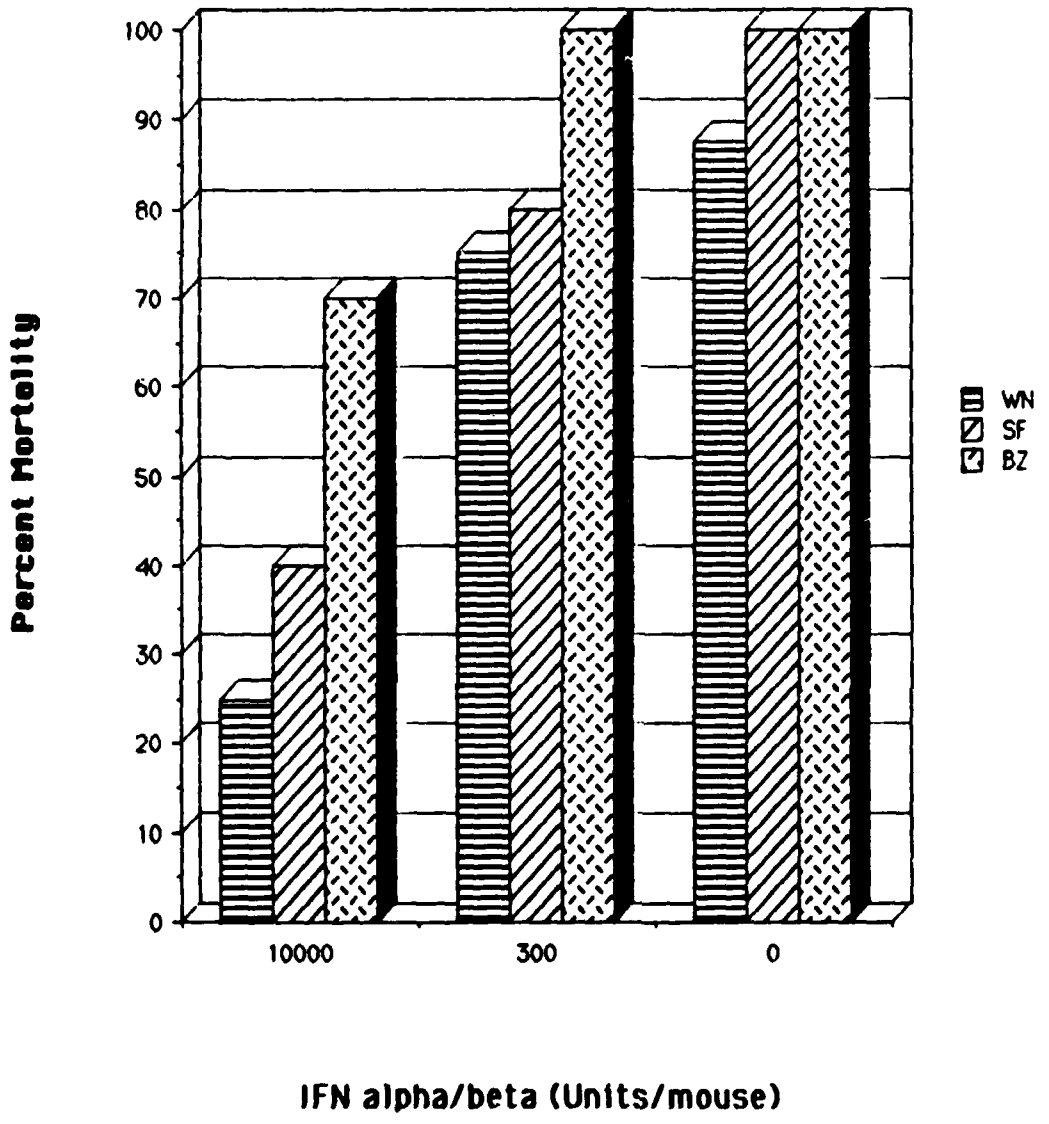
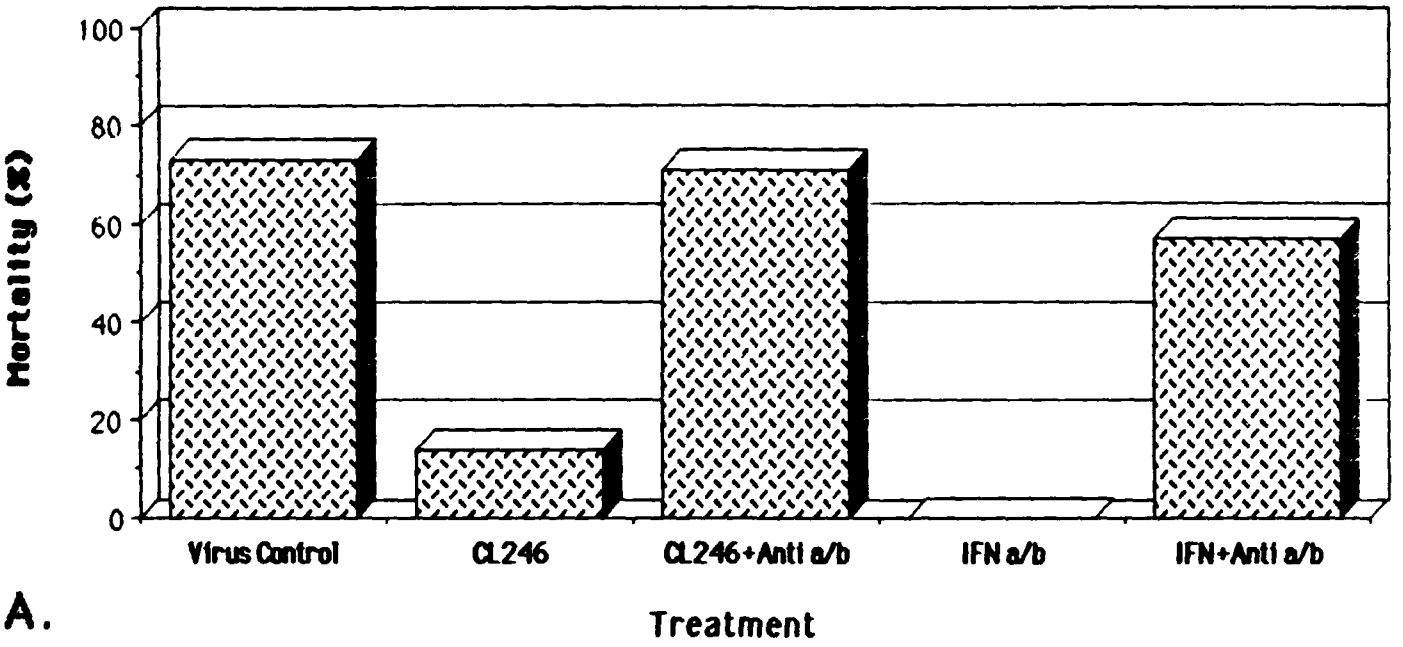


Figure 2. Relative sensitivity of arboviruses to interferon in vivo

Figure 3. Antibodies to IFN Neutralize the Antiviral Action of CL246,738



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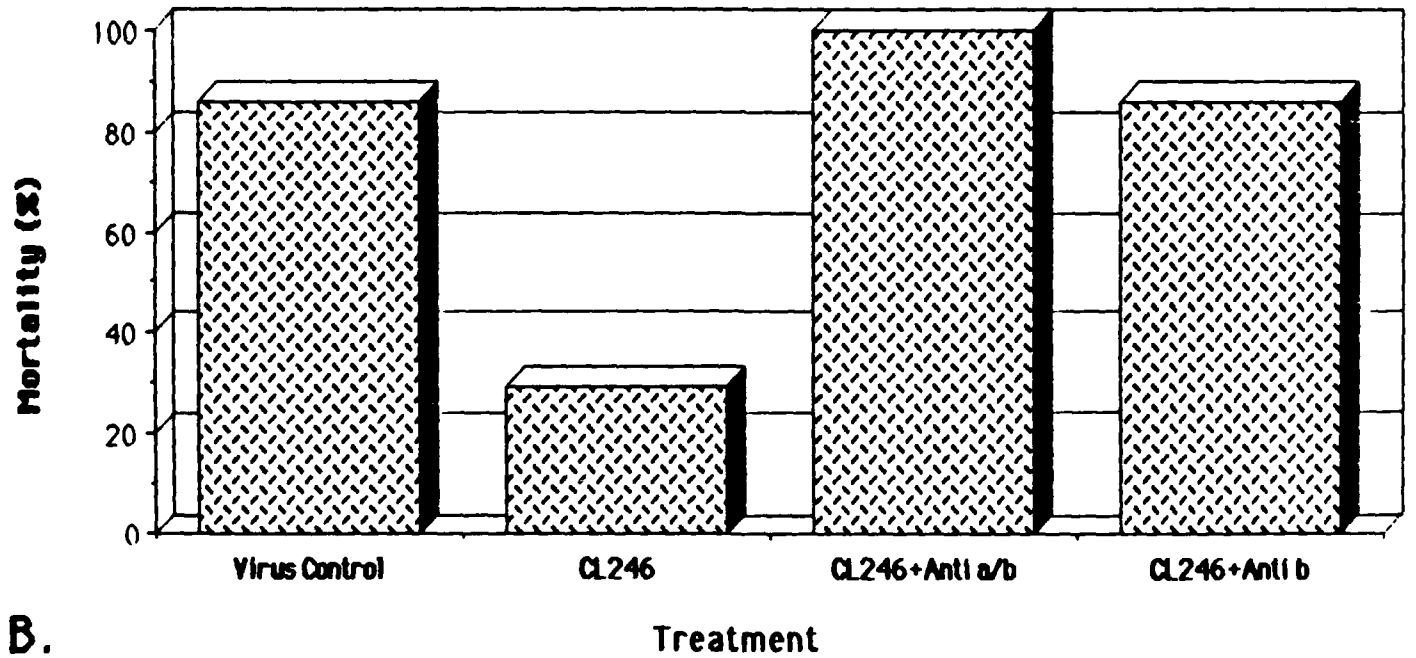

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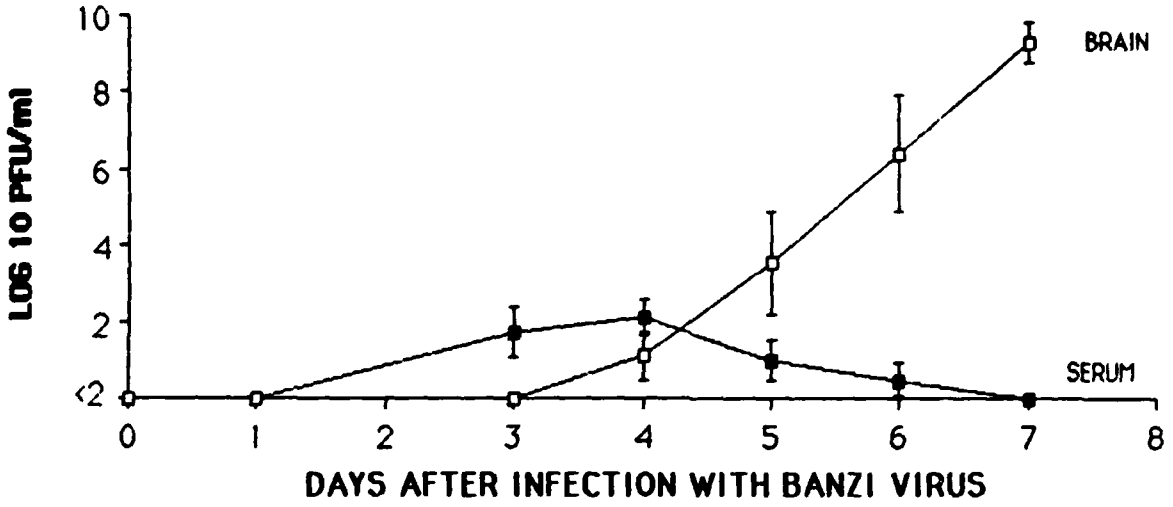


FIGURE 4

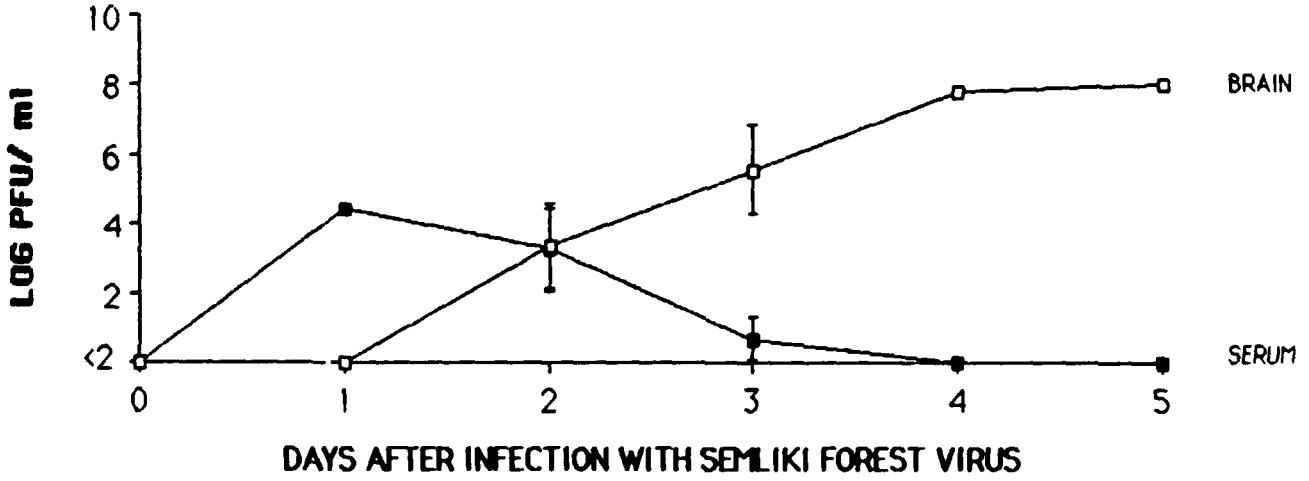
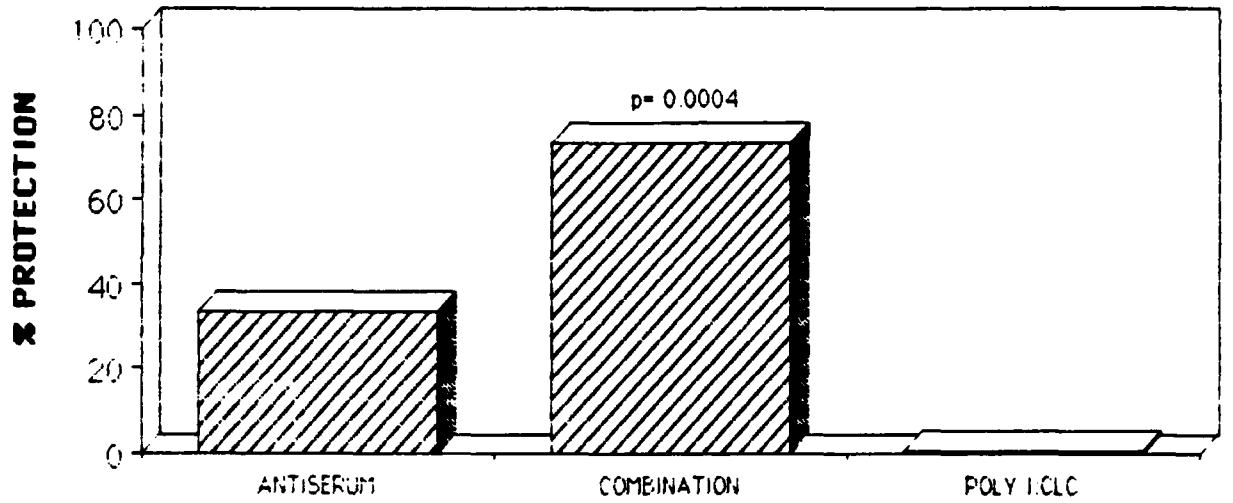


FIGURE 5

Prevention of Banzi Virus Lethal Encephalitis by Late Therapy with Poly I:CLC and Antiserum



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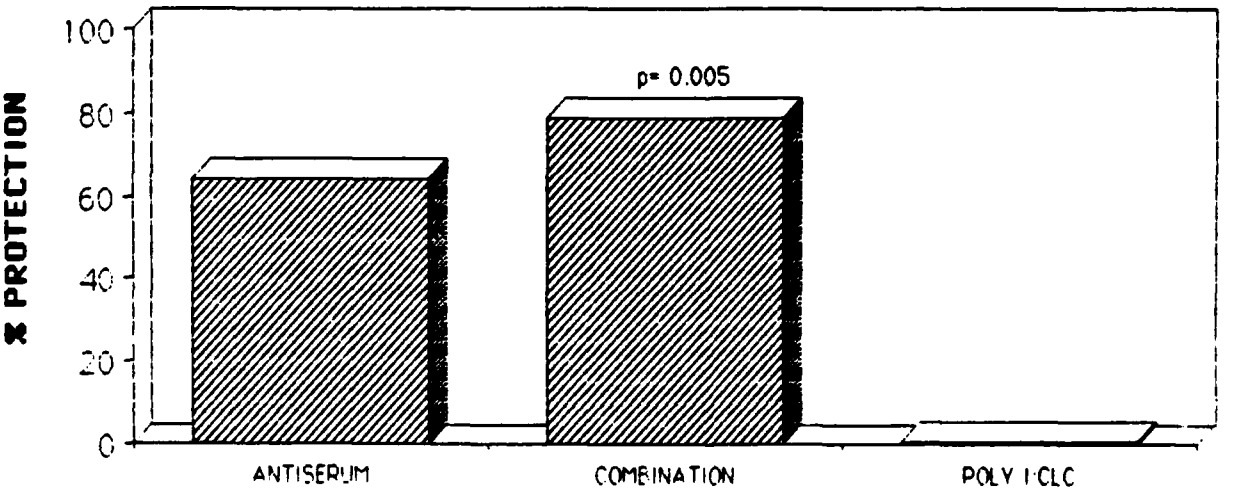

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